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CYTOTOXICITY OF VIRUS

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Helmut Mahnel, Institute
for Microbiology and In-
fectious Diseases of
Animals, Munich University

Abstract

Cytotoxic effects through virus capable of propagation such as those of the pox group inactivated by ultraviolet exposure, were produced under high virus-cell ratios in various cell cultures. In the inoculated culture systems, these effects do not propagate in stages and cannot be transferred to new cultures. The cytotoxic effect was linked to the virus particles. The "changed" cultures do not show any observable virus propagation. Comparison of three members of the pox group, in relation to cytotoxicity and virus propagation in various cultures, indicates that the cells propagate virus to greater or lesser extent or not at all whereas the conditions for cytotoxicity are very probably uniform in all members of the group. There was no correlation between these two virus effects. In regard to virus propagation, the individual types are different from each other in different cell systems but act uniformly in regard to cytotoxicity, independently of the cell system.

Introduction

The first attempt for defining the concept "virus toxicity" or "virus toxin" was made in Ref. 5. The author thus

designated the capability of a virus of damaging cells or tissues, without involving any propagation of virus. However, this definition today is no longer applicable for all toxic effects of virus described.

In principle, we must distinguish between two possibilities of toxic effects of virus. The first type of virus toxin is not linked to the pathogen. With adenovirus, polio virus and the influenza viruses, toxic fractions were separated from the infectious particle (Ref. 1, 10, 18, 19, 24, 26-28, 31, 33). The influenza virus additionally produces a cytotoxic reaction which is apparently based only on an enzymatic damage of the cell and the receptor system of the latter by the virus, i.e. more of a path-breaking disturbance in which the viruses themselves do not have a direct toxic effect on the cell (Ref. 19, 27, 29, 33). This type of toxicity was recently discussed in a synoptic review (Ref. 30).

The second group of toxic damages is produced by the action of large amounts of infectious or inactivated viruses on the cell. In this case, the action is closely linked to the particles. Such observations were made primarily for large virus (Ref. 2-4, 14, 15, 23, 25). The cause is not known. Cytopathological reactions appear to occur only when a virus-cell ratio of a given degree exists.

In regard to the mechanism of action, it was initially believed that the toxic effect must be separated in principle from the propagation of the virus, regardless of the manner of triggering described above. However, more recent findings indicate that this assumption is not necessarily true. Numerous observers of cytotoxic cell reactions, especially in virus of the myxo group, also noted that incomplete cycles of infection, so-called abortive cell infections, took place in the cell (Ref. 9, 11, 17, 20, 32, 34). These are apparently virus infections. However, a production of mature new virus capable of infection takes place in the cell either not at all or only to a very minor extent.

The present communication concerns the cytotoxicity of pox virus triggered by high concentrations of elementary virus particles. The investigation utilized chickenpox virus as model and, as cell system, tissue cultures in which the former

do not propagate. Parallel control tests were made with ultra-violet-inactivated vaccin virus.

Methodology

We utilized the virus strains HP₁, HP₂, HP₇ (henpox), TP₁ (pigeonpox), CVA (Vaccinia) and KP (cowpox). Fresh virus of each strain repeatedly passed through egg was kept available as infected choric-allatonic membrane (CAM). Parallel storage of each pox strain was made from culture virus of the 5th to 10th passage through cultures of embryonal fibroblasts from chickens. With the exception of the HeLa cells (Hamburg strain, Dr. Lennartz), the primary tissue cultures were prepared with the customary method of repeated trypsin enzymolysis. We utilized the following types of cells: HeLa cells, renal epithelium of pigs (ENS), renal epithelium of calves (ENK) and fibroblasts of chicken embryos (FHE). The conservation and virus medium in all tissue cultures was bovine amniotic fluid (RAF) and the vessels used were Roux and Breed-Demeter flasks and test tubes. All cultures were directly inoculated in the conservation medium. In cases where the inoculation medium was removed, we subsequently washed the cespitose growth three times with RAF.

Virus titration was made in FHE-tube cultures with 4 to 6 tubes per dilution. Titers were calculated in KID₅₀ per ml. Virus content of the strain suspensions was determined from two titrations. After obtention from culture vessels by freezing and thawing, the fractions were centrifuged, washed twice with large amounts of RAF and then added to an amount of conservation medium proportional to the culture vessel. The cell-bonded virus was precipitated by shaking with glass pearls or by homogenizing with an "Ultra-Turrax". The cell remains were subsequently removed by centrifuging. The supernatant formed the cellular phase of the virus.

The toxic fractions were obtained from infected CAM. They were harvested at the time of optimum virus content and triturated in the mortar with RAF. The precipitated virus was subsequently purified by fractional ultracentrifuging and concentrated as a high-titer virus suspension. The supernatant of the first ultracentrifuge run, evaluated as soluble

antigen fraction, was again centrifuged for further purification from virus remains, subsequently concentrated twice by careful ammonium-sulphate precipitation, and dialyzed. The second kind of toxic virus preparations were made from the cellular phase of infected FHE cultures. The latter were added to 1/10 of the culture-fluid volume and subsequently hydrolyzed with the "Ultra-Turrax". After removing cell remains by centrifuging, the supernatant constituted the concentrated virus fraction. Additional purification was not made.

Control fractions from non-infected materials (CAM and FHE cultures) were made in the same manner.

We also inactivated concentrated virus suspensions with ultraviolet light. High-titer fractions prepared from FHE cultures not containing phenol red were irradiated by a ultraviolet-light low-pressure source (wavelength at 98% constancy = 254 m/u) as a thin layer in a Petri dish under constant agitation by means of a magnetic agitator. The optimum method for inactivation (curves at constant conditions) had previously been determined from our own experimentation. Random sampling for inactivity was made at a dilution of 1:100 (in order to eliminate interference and dilution of toxicity) in Roux flasks of FHE and undiluted on CAM.

Findings

Cytotoxicity of Henpox Virus in HeLa and ENS Cells

When inoculating high concentrations of henpox virus in HeLa and ENS cell cultures, toxic effects were observed which were not accompanied by a corresponding propagation of virus. The effects could be very rapidly attenuated through dilution of the inoculation material in double stages /"Zweierstufe"/. There was a relatively sharp transition from the final dilution to the ineffective culture. The initial cytotoxicity did not propagate in the culture. Individually affected locations as well as the total culture consequently retained their status after a given instant in time. Removal of the inoculation medium after incubation for six hours regularly increased the initial threshold of the effect.

Cytotoxicity could not be passed to a second culture. It was not possible to reproduce the pictures either by concentrated transfer inoculation of the medium phase or of the hydrolyzed cellular phase. A previous concentration of the phases at 10:1 was also futile.

Cytotoxicity in HeLa Cultures

The first indications appeared at the earliest 2 to 3 days after inoculation. The cells began to become globular with pronounced granulations and signs of shrinking. Some of the cells had assumed a fusiform shape by the 4th day. Fifty percent of the altered cespitose layer then became detached from the glass wall and the remainder was toxicologically altered. Whereas the manifestations described occurred within 1 to 2 days in cultures with high inoculation concentration, with the first signs occurring at the edges of the cespitose layer, this effect was restricted in the dilutions to individual locations, irregularly distributed and not sharply defined.

The cytotoxic effect was different from the specific cytopathogenic effect produced by such virus of the pox group as are capable of propagation and passage such as vaccin and cowpox virus. Here are produced centers of globules as well as giant cells with plasma ramifications and the effect continues until entire destruction of the culture.

Cytotoxicity in ENS Cultures

This differed only very little from the effect of propagation of the vaccin and cowpox virus. The globules were merely not as typical nor the centers as sharp but more diffuse. When most pronounced, the picture consisted of a mixture of globular and granulated cells, of cells with plasma ramifications, and of flaky cell groups and gaps. At a sudden onset after 2 to 3 days, the final state was attained in a few hours and no longer changed after four days (Fig. 1).

Properties of the Toxic Principle

The cytotoxicity was linked to the virus particles. The soluble principle of high-titer virus harvests of CAM was not toxic, even when concentrated at 100:1. All fractions from

non-infected CAM as well as the medium and cell phases of tissue cultures also gave negative results. It also speaks for bonding to the virus that the cytotoxic effect could rapidly be neutralized by dilution /"ausverdunnt" and that entirely analogous preparations, but with a "below-threshold" virus content, had no toxic action. Ultraviolet-inactivated virus suspensions with high initial virus content produced no cytotoxic effect in HeLa and ENS cultures. Toxic fractions incubated with immune chicken serum lost their activity. High-titer virus suspensions purified by fractional ultracentrifuging had, at the same virus content, the same cytotoxicity as non-purified preparations. It was possible to conserve and store the toxic fractions like virus suspensions. With decreasing virus content, cytotoxic potency is also reduced.

Cytotoxicity and Virus Propagation

Table 1 and 2 indicate that a cytotoxic effect could be obtained in 50% of the cells as a rule only after infection by a virus-cell ratio of 1:1 and greater. Smaller inoculation concentrations produced almost no cytotoxic effect.

The virus of henpox was well adsorbed by HeLa cells (Fig. 2 and 3). The virus content in the culture medium dropped by about 80% in this short phase. In a strongly cytotoxic inoculation concentration (Fig. 2), the curves of free virus and cell-bonded virus intersected. However, a rise of the curves never occurred. Cultures with slight or absent toxic effect (Fig. 3) also showed no propagation of virus.

The difference from the control curve may be due to the fact that the cespitoso layer, in contrast to the cell-free medium, stabilizes numerous virus particles. The parallel drop in cultures with weak or absent toxic effect simultaneously indicates that the virus curve has no correlation to the cytotoxic effect. If the medium was removed after high inoculation dose (Fig. 4), we found in the conservation medium a virus content almost decreasing with the control in spite of a slight cytotoxic effect. A slight bend in the curve prior to onset of the cytotoxic effect cannot be interpreted as true virus propagation.

From a comparison of the cytotoxic effect in the various HeLa systems with the virus movements and controls, it becomes clear that the cytotoxic effect is an independent phenomenon. A confrontation with the known curves of virus propagation of pox virus in the cell system makes it evident moreover that it is not necessary to consider virus propagation in our experiments.

Table 1

Kultur- system	(b) Toxische Fraktion (e) (f)			(c) cIE in der Kultur (g) (h)			Virus- Zell-Ver- hältnis bei 50% cIE (d)
	Strain	Her- kunft	Titer -log 10/ml	Incuba- tion	Effekt	(i) Endverd.	
ENS	HP ₇	CAM	8.0	(k) ohne	2./3. Tag 100%	1:8	8:1
		CAM	8.0	mit (1)	3. Tag 50%	1:4	> 16:1
		FHE	8.25	ohne	3./4. Tag 100%	1:4	10:1
	HP ₁	FHE	6.0	ohne	2./3. Tag 50%	1:4	1:1
		FHE	7.5	ohne	2./3. Tag 100%	1:2	3:1
	HP ₂	CAM	7.5	mit	5./7. Tag 50%	1:8	8:1
		CAM	7.5	ohne	5. Tag 100%	1:64	2:1
		CAM	7.0	mit	4./5. Tag 50%	1:4	> 2:1
		CAM	7.0	ohne	3.-5. Tag 100%	1:16	1:2
		FHE	7.25	ohne	3.-5. Tag 50%	1:4	8:1
HeLa	HP ₁	CAM	7.0	mit	ohne	—	—
		CAM	7.0	ohne	4.-5. Tag 100%	1:16	4:1
		FHE	8.5	ohne	3.-5. Tag 100%	1:4	24:1
		FHE	8.1	ohne	ab 2. Tag 50%	1:1	12:1
	HP ₂	CAM	7.7	mit	ohne	—	—
		CAM	7.7	ohne	5. Tag 100%	1:4	4:1
		FHE	7.5	ohne	4.-5. Tag 50%	1:1	> 4:1
		FHE	7.3	ohne	4.-5. Tag 50%	1:1	> 4:1
TP ₁	CAM	7.5	ohne	(m) fraglich	1:1	—	—
	FHE	7.5	ohne	(k) ohne	—	—	—

Cytotoxicity and Virus-Cell Ratio in HeLa and ENS Cultures.

a = culture system; b = toxic fraction; c = cytotoxicity in the culture; d = virus-cell ratio at 50% cytotoxicity; e = strain; f = origin; g = incubation; h = effect; i = terminal dilution; k = without; l = with; m = questionable. Cell systems with 50% cytotoxicity show, at the height of effect, the following virus values (-log₁₀/ml). ENS (3 to 4 days): cellular phase 3.5-4.5; medium 2.5-3.0. HeLa (5-6 days): cellular phase 3.0-5.5; medium 2.0-3.8. n = day

Table 2

Kultur- system (c)	Stamm (d)	(a) überimpfte Fraktion der 1. Passage			(b) 2. Passage		(i) Zellfraktion am 9. Tag -log 10/ml	
		(e) Zellbild bei Entnahme	(f) einge- concentr. gegt. (1)	Titer -log 10/ml	Animpf. Uter im Medium	Virus- Zell- Verh.		
ENS	HP ₁	80% ctE, 3. Tag	10:1	5,1	4,5	1:20	2,1	
	HP ₂	80% ctE, 3. Tag	10:1	5,0	5,1	1:6	2,0	
HeLa	HP ₁	90% ctE, 7. Tag	10:1	5,3	4,6	1:20	4,0	
	HP ₁	100% ctE, 7. Tag	10:1	6,0	5,4	1:2,5	3,0	
	HP ₂	90% ctE, 6. Tag	10:1	5,0	4,5	1:30	n. d.	
	HP ₂	80% ctE 4. Tag	10:1	5,5	4,0	1:10	3,5	
	HP ₂	80% ctE, 4. Tag	10:1	6,25	5,6	1:2	n. d.	

Virus-Cell Ratios in Continued Passage of Yields from Cytotoxic Altered Cultures. All cultures inoculated in the second passage remained ineffective and the inoculation medium was not removed.

a = inoculated fraction of first passage; b = second passage; c = culture system; d = strain; e = cell picture upon sampling; f = concentrated; g = inoculation titer in medium; h = virus-cell ratio; i = cell fraction after nine days; k = not carried through; l = *. The cespitose fraction was absorbed only in one part of the medium volume and then further processed like the toxic virus fractions. m = day

ENS cultures also absorb about 90% of the introduced virus. An eclipse is also recognizable from the comparison of the virus curves of the medium and of the cell phase of the first hours (Fig. 5). In the culture with definite toxic effect, a somewhat better virus stabilization was apparent than in the ineffective culture after a weaker inoculation concentration where the virus curve almost corresponded to the control curve (Fig. 6). Here also, the cytotoxic effect showed no relation to the virus content of the culture.

Virus from toxic-altered cultures no longer propagated even in continued passage (Table 2). A cytotoxic effect could

no longer be obtained in the second passage. The initial cytotoxic effect did not continue in the culture as is always observed in virus propagation in receptive cell systems (propagation cycles). That the virus-cell reaction had an effect in the culture in only one stage, supports the assumption that the cytotoxic effect is dependent on a given initial virus concentration. This relation no longer seems to exist for a second cytotoxic phase.

Cytotoxic Effect by Ultraviolet-Inactivated Virus Suspensions

High-titer virus suspensions of vaccin and henpox virus produced no cytotoxic effect in HeLa and ENS cultures after fresh ultraviolet-inactivation. However, a definite cytotoxic effect could be observed in FHE cultures. The ultraviolet-vaccine virus became effective after 1 to 2 days and, in most tests, destroyed the culture after four days. The cespitose layer dissolved with increasing formation of small granules. Ultraviolet henpox suspensions produced an effect of the same kind but much weaker; the cespitose layer was conserved as a rule and recuperated. A secondary finding was that better and more "interferon" was formed after ultraviolet-virus treatment in cultures with cytotoxic effect than in cell systems without or with weaker cytotoxic effect.

Cytotoxic Effect and Virus Propagation of Other Pox Virus in Different Cell Systems

In working with vaccin, cowpox and henpox virus, we found that these three members of the smallpox group propagated very differently in different cell cultures but that the sequence of intensity vaccin, cowpox, henpox was always followed. We even noted a reduction in the receptivity of the investigated cell cultures (Fig. 7 and Table 3). Whereas the vaccin virus was capable of propagation and passage in all cultures, the virulence of the cowpox virus definitely diminished. It propagated more poorly and slowly and was not capable of continuing passage in ENK cultures. The virus of henpox propagated only in FHE cultures.

Table 3

	(a) Vaccinivirus	(b) Kaposivirus	(c) Ebolavirus
FHE	(d) ctE am Ende des 1. Tages bei 1:1 Virus-Zell-Verhältnis, (kleine Granula) geht in viruspez. Effekt unter. Vermehrung sehr gut, zisch, sekundär bis 100% fortgeschreitend. Vermehrungsrate (+) optimal bis 1:10000.	(e) ctE am 1. Tag bei 1:1 Virus-Zell-Verhältnis, (kleine Granula) geht in viruspez. Effekt über. Vermehrung gut, sekundäre Ausbreitung langsamer als bei Vaccine, bis 100%; Vermehrungsrate optimal bis 1:1000. ctE nicht erkennbar, da die Vermehrung sehr rasch anläuft und fortgeschreitet. Vermehrungsrate optimal etwa 1:1000.	(f) ctE am 2.–3. Tag bei etwa 5:1 Virus-Zell-Verhältnis, kleine Kugeln und ausgedehnte Granula, Zellrasen kann am 3. Tag in toto abgehen. Vermehrung noch gut, sekundär sehr langsam, aber bis 100% fortgeschreitend. Vermehrungsrate optimal 1:100.
HeLa			(g) ctE am 1. Tag bei 1:1 Virus-Zell-Verhältnis; scholliger und Abkugelungs-Effekt. Vermehrung schlecht, sekundär sehr langsam, z. T. ausbleibend. Vermehrungsrate optimal 1:10.
ENS		(h) ctE bei 1:1 Virus-Zell-Verhältnis am 1.–2. Tag; scholliger Zerfall mit Kugelungen. Vermehrung mäßig, langsam, optimal bis Rate 1:10, fortfallende Passagen nur bei aufsechterhaltbarem Mindesttitrermöglich.	(i) ctE am 2.–3. Tag bei etwa 5:1 Virus-Zell-Verhältnis am 2.–3. Tag; schollig und granulär am 2.–3. Tag. Vermehrung nachweisbar, keine echte Vermehrung . Keine laufenden Passagen möglich.
ENK		(j) ctE am 1. Tag bei 1:1 Virus-Zell-Verhältnis; scholliger Zerfall und Granula. Vermehrung mäßig bis gut, Vermehrungsrate um 1:1; Passagen reißen rasch ab.	(k) ctE bei 5:1 Virus-Zell-Verhältnis, (P) granulärer Reaktion bei etwa 1:1 Virus-Zell-Verhältnis. Vermehrung schlecht, Vermehrungsrate um 1:1; Passagen reißen rasch ab.

Comparison of Cytotoxicity and Virus Propagation of Vaccin, Cowpox and Henpox Virus in FHE, HeLa, ENS and ENK Cultures.

Table 3

Comparison of Cytotoxicity and Virus Propagation of Vaccin, Cowpox and Henpox Virus in FHE, HeLa, ENS and ENK Cultures.

a = vaccin virus; b = cowpox virus; c = henpox virus; d = cytotoxicity after 24 hours at virus-cell ratio 1:1, (small granules) obscured by virus-specific effect. Propagation very good, rapid, secondary progression to 100%. Propagation rate (+) optimum to 1:10,000; e = cytotoxicity on first day at virus-cell ratio 1:1, (small granules) transformed to virus-specific effect. Propagation rate optimum 2 to 1,000; f = cytotoxicity after 2 - 3 days at virus-cell ratio about 5:1, small globules and extensive granules, cespitose layer may detach totally on third day. Propagation still good, secondary propagation very slow but continue to 100%. Propagation rate optimum to 1:100; g = cytotoxicity not recognizable because propagation starts quickly and progresses. Propagation rate optimum to about 1:1,000; h= cytotoxicity on first day at virus-cell ratio 1:1; flaking and blobulating effect. Propagation poor, secondary progression very slow and occasionally absent. Propagation rate optimum to 1:10; i = cytotoxicity between 3 to 5 days at virus-cell ratio of about 5:1. No demonstrable propagation. No continuing passages possible; k = cytotoxicity not observed. Propagation very good, rapidly progressing; propagation rate optimum to about 1:10,000; l = cytotoxicity between 1 and 2 days at virus-cell ratio 1:1; flaky decomposition with globulation. Propagation moderate, slow, optimum to 1:10. Continuing passages possible only when minimum titer can be maintained; m = cytotoxicity after 2 to 3 days at virus-cell ratio of about 5:1; flaky and globular decomposition. No demonstrable propagation. No continuous passages possible. n = cytotoxicity on first day at virus-cell ratio 1:1; flaky decomposition and granules. Propagation moderate to good, propagation rate optimum to about 1:100; o = cytotoxicity after

1-2 days with flaky, granular reaction at a virus-cell ratio of about 1:1. Propagation poor, propagation rate about 1:1; passages are rapidly disrupted; p = cytotoxicity at virus-cell ratio 5:1, flaky and granular after 2-3 days. No true propagation demonstrable, no passages possible; q = (+) rate of propagation signifies the ratio between inoculation and yield of virus of the total culture.

However, in almost all pox virus and in all types of cell cultures, a cytotoxic effect was observed under concentrated inoculation.

This becomes perceptible only under extremely close observation with a well and rapidly growing vaccin virus and is recognizable only by the fact that its type deviates from the normal effect and cannot be explained by the propagation curve. In the other two pox virus, the cytotoxic effect is more clearly differentiable from the propagation effect since the latter occurs later and the propagation cannot be correlated to the intensity of the cytotoxic effect.

Discussion

Observations on toxic action of pox virus have so far been made primarily for the vaccin virus (Ref. 2, 12-15, 25). Our findings with henpox virus complement this possibility of action of the pox virus on cells and agree with earlier findings. It was also demonstrated concurrently that the capability for cytotoxic action is linked to the virus particles.

Moreover, all authors note that a high virus-cell ratio is prerequisite for toxic action in the infection. Our findings agree relatively well with these indications (Ref. 12-14), and even with findings for other types of virus (Ref. 16).

The cytotoxic effect dependent on high inoculation concentration could be observed in several kinds of cells. The initial conditions remained within very narrow limits, although the pox virus propagated either very differently or not at all in these different cell systems.

The cytotoxic effect produced in HeLa and ENS cultures by henpox virus was not capable of passage. The toxically altered cultures did not form any new virus. Transfer inoculation does not produce the initial concentration required for the cytotoxic effect. Locally occurring cytotoxic effect does not propagate in the culture and thus remains monophase. The same observation was made for vaccin virus in cultures of embryonal mice cells (Ref. 14).

In locally occurring cytotoxic effect, the time of onset was so delayed that we may possibly assume a simultaneous "interferon" action as inhibiting the effect. Indications for such an action are suggested. In virus of the myxogroup (NDV, Sendai virus), such correlation between toxic effect and "interferon" has already been determined (Ref. 14-16).

Cytotoxic effects could also be produced with concentrated and ultraviolet-inactivated suspensions of pox virus. Indications of the same kind also exist (Ref. 2). A very recent communication confirms our findings with vaccin virus after terminating our own investigation (Ref. 12, 13). When ultraviolet-inactivated, the easily propagating vaccin virus has a greater toxic action than the henpox virus. A secondary finding were certain parallels in the relatively satisfactory "interferon" formation. That the interferon itself here acts toxic should as yet not be assumed, although such assumptions already exist (Ref. 5).

Recent investigations have confirmed that the pox virus -- like other virus -- are absorbed by the cell in accordance with the type of cytosis and are then decomposed (Ref. 6-8, 21, 22). It is probable that the decision for capability of reproducing the pathogen is made in the cell only subsequently. We do not yet know whether all types of cells, even those "non-receptive", are able to absorb in this manner pox virus capable of propagation or inactivated. If we assume this, an explanation of the toxic action due to the "unrestricted" absorption of many or of few as well as also inactivated virus particles is more easily conceivable. The virus with demonstrably analogous mechanism of infection (smallpox, NDV, Sendai virus) show parallels also in the cytotoxic effect.

The entire observations with virus of the pox group in different cell systems make it probable that such virus encounters, in regard to virus propagation, either receptive -- more or less -- or non-receptive cells. Non-receptive here does not signify that no virus enters the cell. In orienting electron-optical investigations we were able to demonstrate that large numbers of ultraviolet-inactivated particles are also absorbed and decomposed by the cell. However, we may assume from present findings, that virus propagation in the cell becomes clearly indicated beyond a given "threshold of receptivity" and that no virus synthesis takes place below this threshold. With high infectious doses, a toxic action probably occurs independently of this. Its cause could not yet be clarified.

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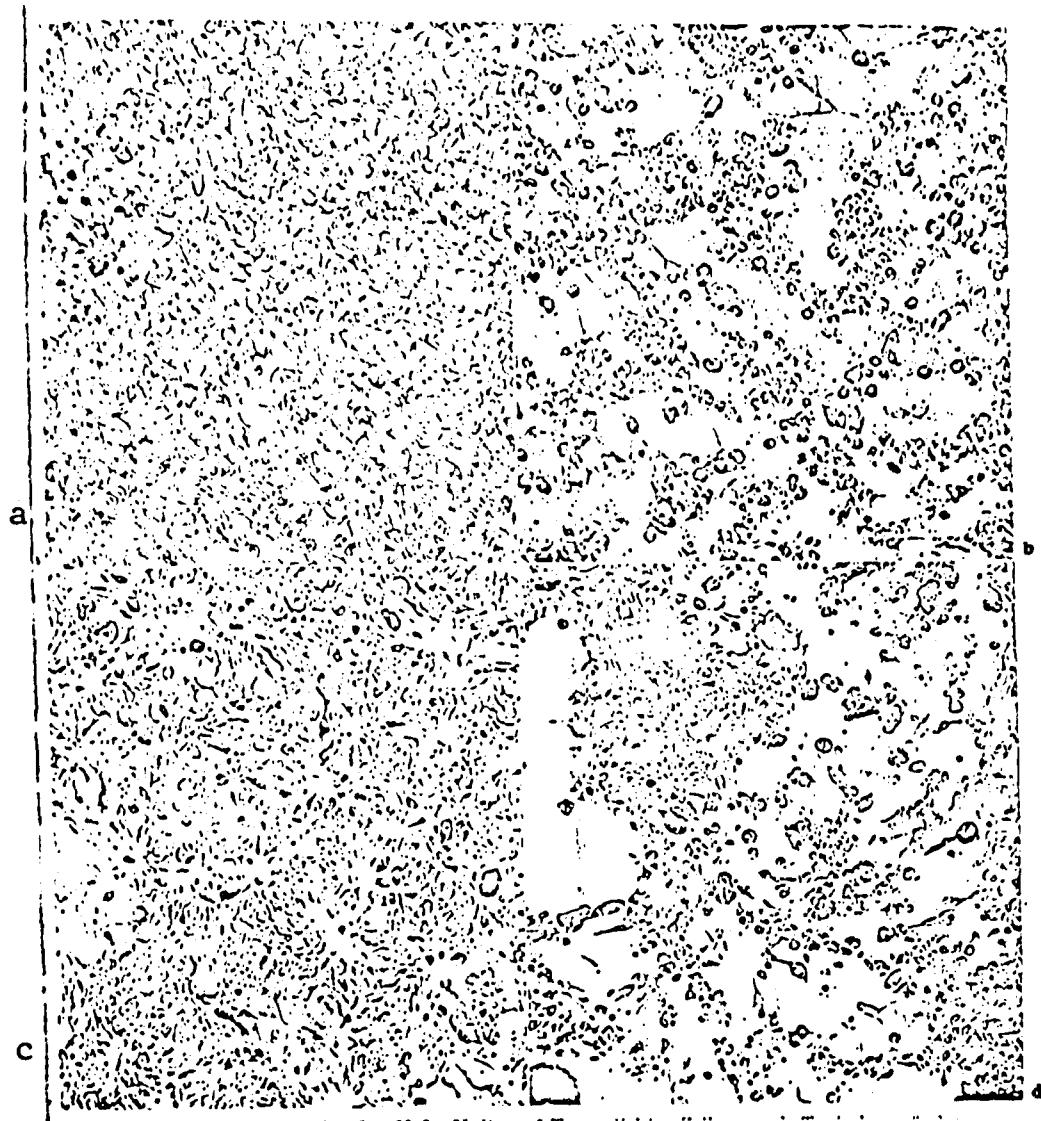


Figure 1. a) Normal Non-inoculated HeLa Culture, Dense Cespitose Layer after Nine Days; b) Toxic Altered HeLa Culture Five Days after Inoculation with HP₇; c) Normal Non-inoculated ENS Culture, Dense Cespitose Layer after Seven Days; d) Toxic-Altered ENS Culture Four Days after Inoculation with HP₂.

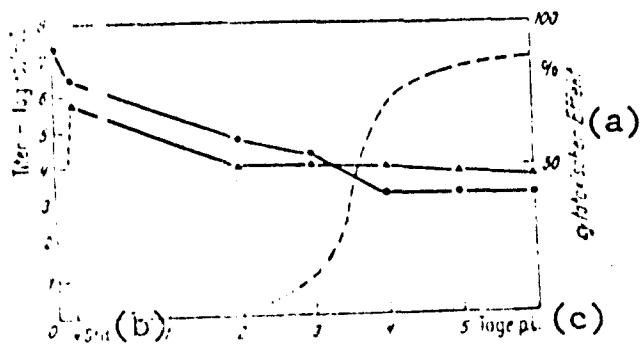


Figure 2. HeLa Culture. Infected by HP₁ without Change of Medium. Virus Concentration in Medium (Dot) and Cellular Phase (Triangle). Broken Line = Cytotoxic Effect. a = cytotoxicity; b = hours; c = five days after infection.

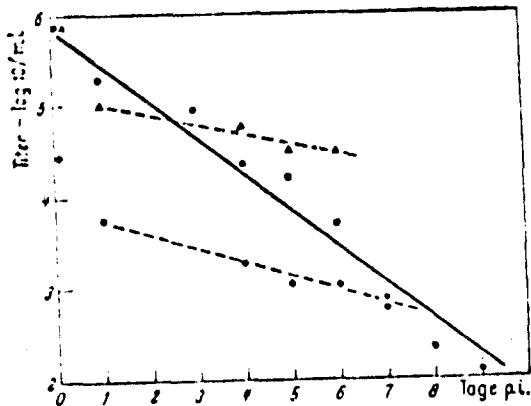


Figure 3. HeLa Culture, Infected with HP₁ without Change of Medium. Virus Content in Medium after Toxic (Triangle) and Non-toxic (Diamond) Inoculation Dose and Virus Control in Cell-Free Medium (Dot)

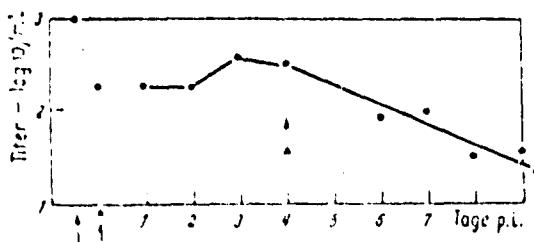


Figure 4. HeLa Culture, Infected with HP₁. Inoculation Medium Removed After Six Hours with Three Subsequent Washings. Virus Content in Medium After Inoculation Concentration of $10^{7.2}/\text{ml}$ Titer at Next to Last (Arrow) and Last (Arrow and Superposed Triangle) Washing. Feathered Arrow = Onset of Cytotoxic Effect of 40%

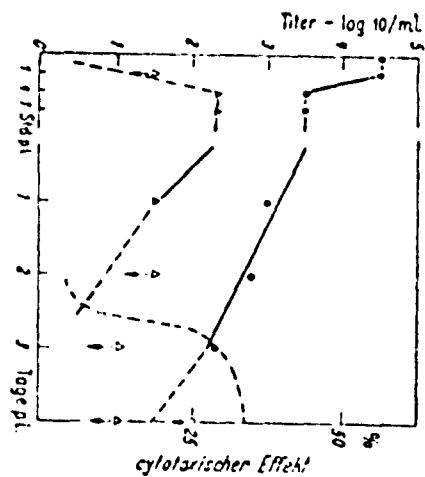


Figure 5. ENS Culture Infected with HP₁ without Change of Medium. Virus Concentration in Medium (Dot) and Cellular Phase (Triangle) as well as Cytotoxic Effect (Broken Line). Open Triangle = Negative Specimen

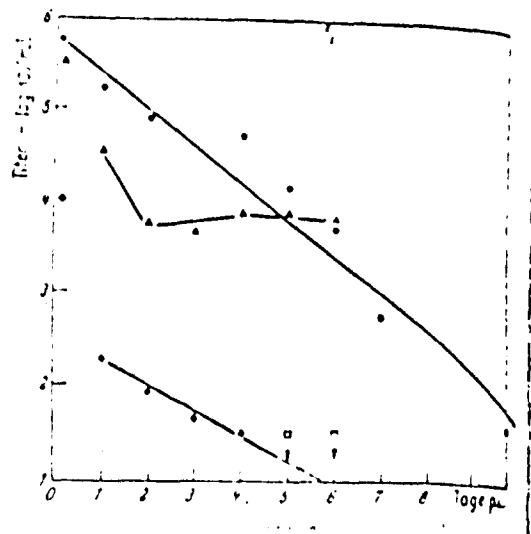


Figure 6. ENS Culture Infected with HP₁ without Change of Medium. Virus Content in Medium after Toxic (Solid Triangle) and Non-toxic (Solid Diamond) Inoculation Dose as well as Virus Control in Cell-Free Medium (Solid Dot). Open Square = Negative Specimen.

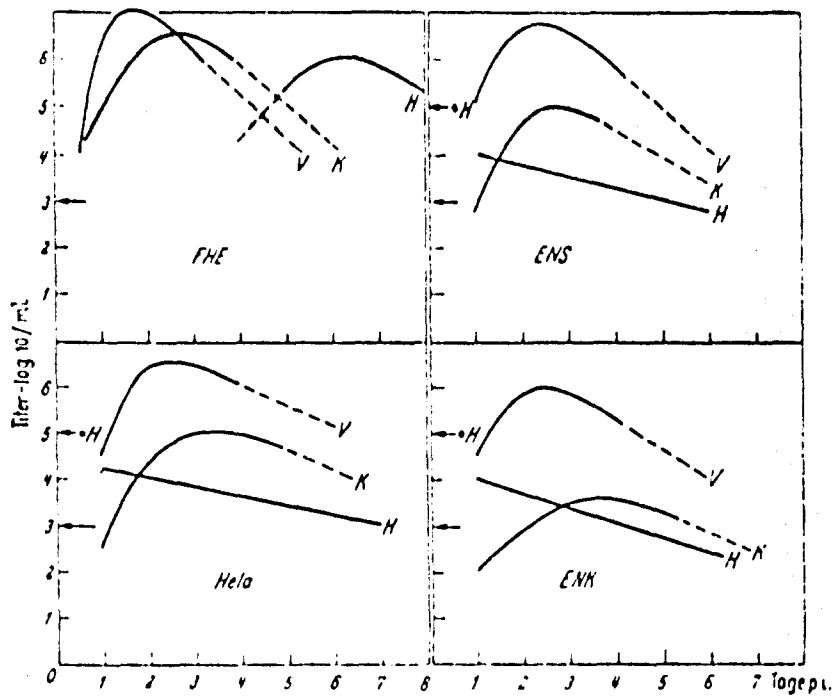


Figure 7. Comparative Schema of Average Propagation Curves of Vaccin (V), Cowpox (K) and Henpox (H) Virus in Different Cell Cultures (Total Virus). Arrow = Inoculation Concentration in Medium.